

Common Mutations in the Fibroblast Growth Factor Receptor 3 (FGFR 3) Gene Account for Achondroplasia, Hypochondroplasia, and Thanatophoric Dwarfism

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The mapping of the achondroplasia locus to the short arm of chromosome 4 and the subsequent identification of a recurrent missense mutation (G380R) in the fibroblast growth factor receptor 3 (FGFR-3) gene has been followed by the detection of common FGFR-3 mutations in two clinically related disorders: thanatophoric dwarfism (types I and II) and hypochondroplasia.

The relative clinical homogeneity of achondroplasia was substantiated by demonstration of its genetic homogeneity as more than 98% of all patients hitherto reported exhibit mutations in the transmembrane receptor domain. Although most hypochondroplasia cases were accounted for by a recurrent missense substitution (N540K) in the first tyrosine kinase (TK 1) domain of the receptor, a significant proportion (40%) of our patients did not harbor the N540K mutation and three hypochondroplasia families were not linked to the FGFR-3 locus, thus supporting clinical heterogeneity of this condition.

In thanatophoric dwarfism (TD), a recurrent FGFR-3 mutation located in the second tyrosine kinase (TK 2) domain of the receptor was originally detected in 100% of TD II cases, our series seven distinct mutations in three different protein domains were identified in 25 of 26 TD I patients, suggesting that TD, like achondroplasia, is a genetically homogenous skeletal disorder.

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KEY WORDS: FGFR-3, achondroplasia, hypochondroplasia, thanatophoric dysplasia, tyrosine-kinase

INTRODUCTION

More than 10 years ago, J. Spranger proposed the existence of chondrodysplasia phenotype "families" of differing severity on the basis of common clinical and radiological manifestations [Spranger, 1988]. One of these, the achondroplasia family, is characterized by a continuum of severity ranging from mild forms (hypochondroplasia) to lethal neonatal dwarfism (thanatophoric dysplasia). It was then hypothesized that mutations in a common gene could account for these conditions. This assertion was supported by several observations: first, inter- and intra-familial variability does exist in hypochondroplasia with some forms resembling achondroplasia; second, a proband born to a parent with achondroplasia and a parent with hypochondroplasia had more severe skeletal abnormalities than individuals with heterozygous achondroplasia; and third, homozygous achondroplasia patients born to heterozygous parents usually died at birth or soon after with radiological changes closely resembling TD.

In 1994, a gene for achondroplasia was mapped to 4p16 [Le Merrer et al., 1994; Velinov et al., 1994]. Among the five genes that had been ascribed to this 2.5 Mb region, the FGFR-3 gene, which is expressed in cartilage [Peters et al., 1993], appeared as the best candidate. This assertion was rapidly substantiated by detection of a recurrent FGFR-3 mutation in 39/39 achondroplasia patients [Shiang et al., 1994; Rousseau et al., 1994b].

Consistent with the relative clinical homogeneity of achondroplasia was the demonstration that more than 97% of all patients had the G380R substitution in the transmembrane receptor domain, thus indicating that guanosine 1138 of the FGFR-3 gene might represent the most mutable nucleotide in the genome [Bellus et al., 1995a]. Not unexpectedly, FGFR-3 mutations were de-

Received for publication January 3, 1996.

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Dedicated to Jürgen W. Spranger on the occasion of his 65th birthday with admiration and best wishes.

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tected in both types of TD [Tavormina et al., 1995b; Rousseau et al., 1995], either in the extracellular or intracellular domains of the receptor. Finally, a recurrent missense mutation located in the TK 1 receptor domain was recently shown to account for more than 60% of hypochondroplasia cases, thus confirming that achondroplasia and hypochondroplasia are allelic disorders [Bellus et al., 1995b; Prinos et al., 1995].

Identification of FGFR-3 mutations in additional achondroplasia patients, in 23 familial or sporadic cases of hypochondroplasia, and in a series of 25 cases with TD I is reported here. Radiological manifestations of TD I patients harboring mutations in three distinct domains of the receptor are presented.

MATERIALS AND METHODS

Patients and Cultured Cells

Blood samples were collected from achondroplasia and hypochondroplasia patients. Skin biopsies and cartilage samples from aborted TD fetuses were obtained with agreement of the family and pregnancy termination. Genomic DNA was extracted according to standard procedures from white blood cells, dermal fibroblast cultures, or frozen tissues. For histological studies, decalcified sections were stained with hematoxylin, eosin, and safran.

PCR Amplification and SSCP Analysis

Exon numbering was based on the genomic organization of the mouse *Fgfr-3* gene [Perez-Castro et al., 1995]. Exons 10, 11, and 19 were amplified by using primers presented in Table I. Polymerase chain reaction with annealing temperature of 62°C for 35 cycles in the presence of 5% DMSO generated PCR fragments of 186 bp, 180 bp, and 226 bp. Amplification of exon 7 which encodes the linker region between the Ig II and III loops was achieved by using a two-step protocol. Primers are presented in Table I and PCR conditions were similar to those described by Tavormina et al. [1995a], which resulted in a 55 bp DNA fragment. For SSCP analysis, PCR was carried out in the presence of [33P] dCTP (0.1 µl/50 µl). Labelled PCR products were

denatured, and then loaded on hydrophobic MDE gels as recommended by the manufacturer (Bioprobe Systems, Paris, France) and run in 0.6 TBE buffer at 250 V for 16 hours. Gels were dried and autoradiographed.

DNA Sequencing and Restriction Enzyme Analysis

PCR products were purified on Nu-Sieve agarose gels and sequenced using the fluorescent dideoxy-terminator method on an automated DNA sequencer (Applied Biosystems, Cheshire, UK). To detect the C/T transition at nucleotide 742 in exon 7, a 55 bp fragment was digested with 5 U of Bsi HKAI (New England Biolabs, Beverly, MA) as recommended by the manufacturer, and then run on a 15% polyacrylamide gel and stained with ethidium bromide. Cleavage into two fragments of 26 and 29 bp was observed when the mutation was present. The C/G transversion at nucleotide 746 created a new BsoFI site which cleaved the 55 bp products into two fragments of 26 and 29 bp. The stop codon mutations at nucleotide 2416 (T/G and T/A) destroyed an MaeII restriction site while the A/T transversion at nucleotide 2418 created an Afl III restriction site.

RESULTS

Detection of Mutations in FGFR-3 Gene by SSCP Analysis and PCR Sequencing

DNA fragments resulting from exon amplification by PCR were either analyzed by SSCP or sequenced directly. Several abnormal patterns were observed in exon 10 which encodes the transmembrane domain (Fig. 1A). The most common pattern (lane 2) corresponded to the previously reported G/A transition at nucleotide 1138 that occurred in achondroplasia patients. It was found in 48/50 cases (Table II); two other patients had a G/C transversion at the same nucleotide. Both mutations resulted in the G380R substitution while the G375C mutation [Superti-Furga et al., 1995] was not detected in our series.

Another abnormal but distinct pattern of exon 10 (Fig. 1A, lane 5) was also detected in 8/26 patients with TD I. Sequencing of the PCR products showed a recurrent A/G transition at nucleotide 1118 which converted tyrosine 373 into cysteine (Table II). In another TD I patient who presented a normal SSCP pattern of exon 10, direct sequencing documented a G/T transversion at nucleotide 1108 converting glycine 370 into cysteine. Finally, a relatively uncommon change was observed in exon 10 in one TD I patient and two control DNAs (not shown). Thymidine 1150 was converted into cytosine resulting in the replacement of phenylalanine 384 by leucine, another hydrophobic residue. Indeed, in the chicken FGFR-3 receptor, residue 384 also is a leucine, thus indicating that nucleotide 1150 substitution should be regarded as a neutral polymorphism (frequency 0.04).

SSCP analysis of exon 11 which encodes the TK I domain showed two distinct patterns (Fig. 1B) that differed from control. They corresponded to either a C/A or a C/T transversion at nucleotide 1659, both substitutions resulting in the replacement of an asparagine residue by a lysine at position 540 of the protein. This

TABLE I. Primers Used for PCR Amplifications

Exon 7: first step:

5' AACCCCACTCCCTCCATCTC 3'
5' CAAGGAGAGAACCTCTAGCT 3'

second step:

5' CTGAGCGTCATCTGCCCC 3'
5' CGCCTGCAGGATGGGCCG 3'

Exon 10

5' GCCGAGGAGGAGCTGGTGGAGGCT 3'
5' CTTGAGCGGGAAGCGGAGATCTTG 3'

Exon 11

5' GCCACTGACAAGGACCTGTCCG 3'
5' CCCTTGCCCGCTACTCCACCAG 3'

Exon 19

5' CCTGTCCGCGCCTTTCGAGCAGTAC 3'
5' CACCAGCAGCAGGGTGGGCTGCTAG 3'

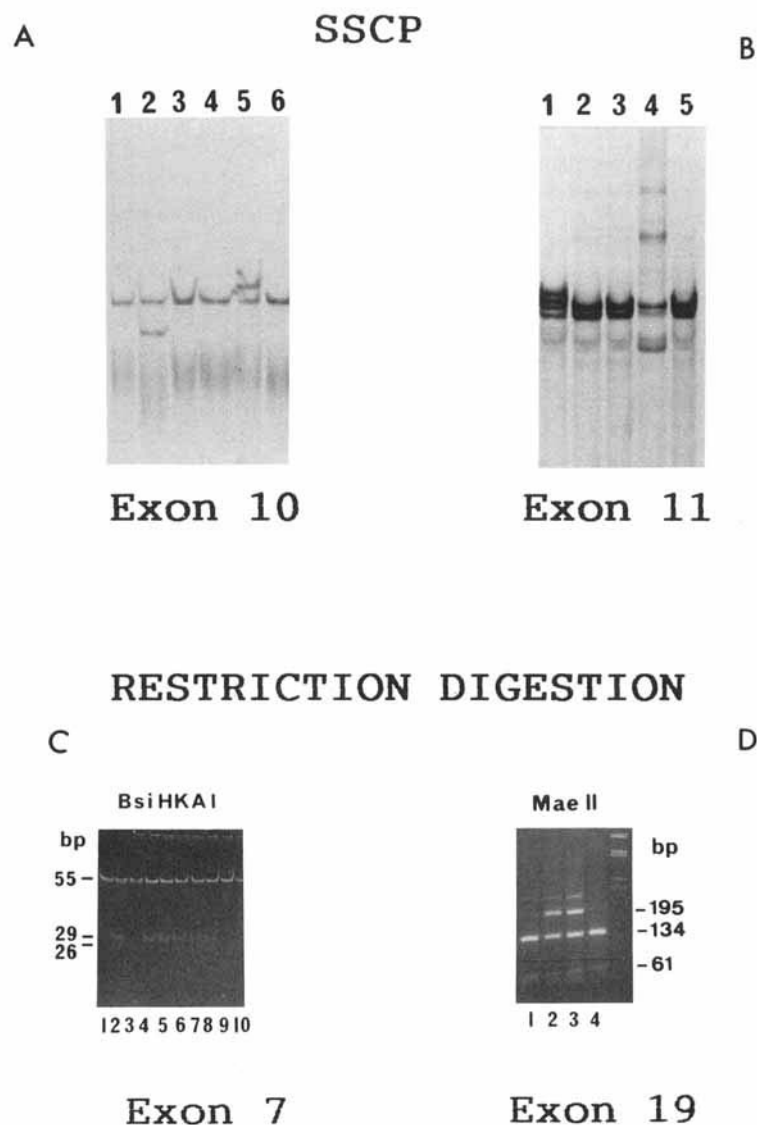


Fig. 1. Detection of FGFR-3 mutations by SSCP and restriction digestion analyses. **A:** Lanes 1, 3, 4, 6, control DNAs; lane 2, achondroplasia patient DNA; lane 5, TD I patient DNA. **B:** Lanes 2, 3, 5, control DNAs, lanes 1, 4, hypochondroplasia patients. **C:** Lanes 1, 10, control DNAs, lanes 2–9, TD I patients (DNAs in lanes 3 and 9 were not digested by Bsi HKAI but by Bso FI which recognized the C746G substitution). **D:** Lane 1, control DNA; lanes 2, 3, 4, TD I patient DNAs (the patient in lane 4 had a base substitution at nucleotide 2418 which did not abolish the Mae II restriction site). PCR products were restriction digested with Bsi HKAI (C) or Mae II (D).

relatively common change was detected in all affected individuals in nine families but was not found in nine other families, three of them showing clear exclusion of the FGFR-3 locus [Rousseau et al., 1994a]. Among 19 sporadic cases, the N540K mutation was observed in 13 individuals (Table II). A third abnormal SSCP pattern of exon 11 was detected in one hypochondroplasia patient who did not carry the common mutation. Sequencing disclosed a silent mutation at position 549 in a glycine codon. Further analysis of parental DNA demonstrated that the asymptomatic father was homozygous for the base change (G/T) which was detectable by restriction analysis with Apa I. Loss of the Apa I restriction site was also detected in 3/40 control

DNAs, thus indicating that this polymorphism was not related to the phenotype.

Restriction digestion analysis of PCR products with Bsi HKAI (Fig. 1C) demonstrated that 9 TD I cases of 26 had a C/T transition at nucleotide 742, which converted arginine 248 into cysteine. In 2/26 cases, a C/G transversion occurred at nucleotide 746 (Table II). Serine 249, like the adjacent arginine 248, was converted into cysteine. Mutations in the stop codon of FGFR-3 were identified in 5/26 patients. Substitutions at nucleotide 2416 (T/G or T/A) abolished an Mae II restriction site in 4 cases (Fig. 1D), while the A/T transversion at nucleotide 2418 created an AfIII site in one patient. Consistent with the clinical diagnosis of TD 1, none of

TABLE II. Summary of FGFR-3 Mutations in Our Series of Achondroplasia, Hypochondroplasia, and TD Patients

Disorder	Number of patients			FGFR-3 mutations		
	Familial	Sporadic	Total	Familial	Sporadic	Total (%)
Achondroplasia	6	44	50			
G1138A				6	42	96
G1138C				0	2	4
Hypochondroplasia	19	19	38			
C1659A				4	9	34
C1659G				4	6	26
TD I	0	26	26			
C742T				0	9	34.6
C746G				0	2	7.7
A1118G				0	8	30.7
G1108T				0	1	3.8
T2416G				0	2	7.7
T2416A				0	2	7.7
A2418T				0	1	3.8

our patients exhibited the K650E substitution that accounts for TD II but an uncommon polymorphism was detected in intron 14 in two TD cases. A summary of FGFR-3 mutations and their relative location in the protein is presented in Figure 2.

Radiological and Histological Data

Radiographs from TD I fetuses carrying three distinct mutations were compared (Fig. 3). Even though femur curving seemed more obvious in some cases, no direct correlation between position of mutations and radiological abnormalities could be established. Cloverleaf skull was present in only one case but abnormal lobulation of the brain and heterotopia of the temporal lobes was seen constantly.

Histological sections of cartilage samples showed short and disorganized chondrocyte columns in the growth plate, reduced number of hypertrophic cells, and lateral overgrowth of bones. Three cases harboring a mutation in the linker region between Ig loops (R248C or S249C) were available for histological analysis. In all of them, a severe disruption of the growth

plate with almost no chondrocyte columns was observed (Fig. 4), suggesting that these mutations might have the most deleterious consequences on cartilage organization. Further cases will be required to verify this assertion.

DISCUSSION

Fibroblast growth factor receptors (FGFRs) comprise a family of four tyrosine kinase receptors [Johnson and Williams, 1993] that bind, with varying affinity, fibroblast growth factors (FGFs). This family of nine structurally related, heparin-binding polypeptides plays a key role in the growth and differentiation of various cells of mesenchymal and neuroectodermal origin [Givol and Yayon, 1992]. FGFs are also implicated in chemotaxis, angiogenesis, programmed cell death, and spatial patterning [Burgess and Maciag, 1989; Basilico and Moscatelli, 1992; Laufer et al., 1994].

Recently, mutations in three domains of the FGFR family were found in several forms of bone dysplasia and craniofacial defects with limb abnormalities [Park et al., 1995; Muenke and Schell, 1995]. The first clues

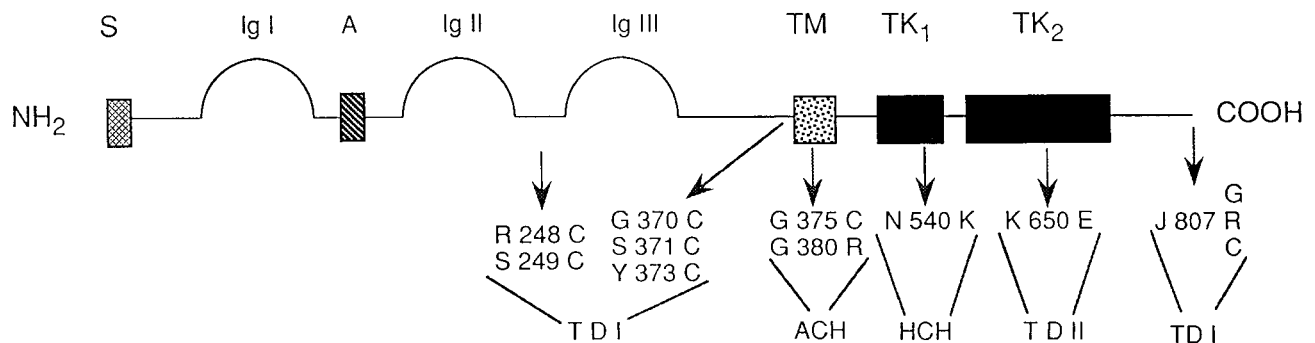


Fig. 2. Schematic structure of human FGFR-3 protein and locations of FGFR-3 mutations causing three skeletal dysplasias: Achondroplasia, hypochondroplasia, and TD (types I and II). TM, transmembrane domain; TK, tyrosine kinase domains (1 and 2); Ig, immunoglobulin-like domains (I, II, III). Arrows show the relative positions of mutations.

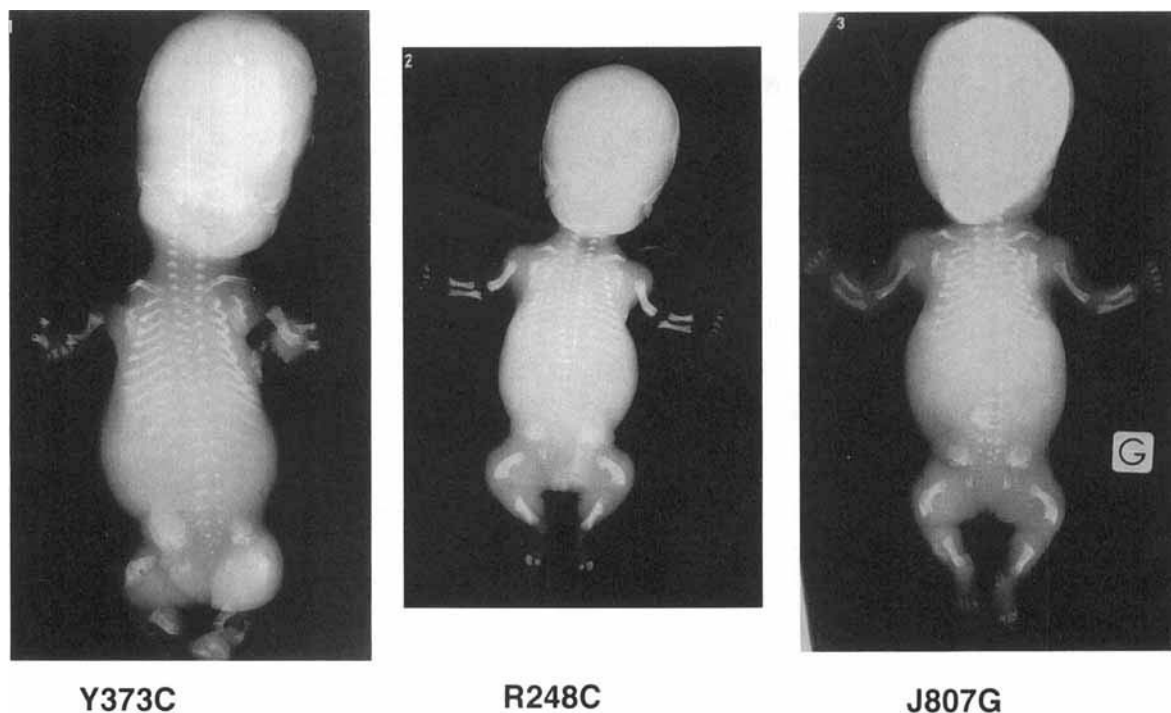


Fig. 3. Radiographs of three TD1 patients carrying mutations in three different FGFR-3 domains. Foetus 1 (19 weeks); foetus 2 (24 weeks); foetus 3 (21 weeks).

of FGFR involvement in chondrodysplasias came from the mapping of achondroplasia to chromosome area 4p16 [Le Merrer et al., 1994; Velinov et al., 1994]. This primary step was followed by the detection of a recurrent mutation in the FGFR-3 gene in achondroplasia patients [Shiang et al., 1994; Rousseau et al., 1994b]. The subsequent identification of FGFR-3 mutations in TD and hypochondroplasia clearly confirms that these three skeletal disorders belong to a phenotypic chondrodysplasia "family" [Spranger, 1988].

Data presented in Table II indicate that achondroplasia and TD are genetically homogenous conditions. A strikingly different situation was observed with other FGFR related defects. Hence, in some types of craniosynostosis (Crouzon and Pfeiffer syndromes), either mutations in the FGFR-2 or the FGFR-1 receptors resulted in the same phenotype [Rutland et al., 1995; Schell et al., 1995].

The mechanisms leading to skeletal growth retardation of variable severity in achondroplasia, hypochondroplasia, or TD are far from being understood, especially as mutations in different critical domains of the receptor have been identified. Binding of a FGF ligand to its receptor is known to require the presence of a low affinity heparan sulfate proteoglycan co-receptor [Spivak-Kroizman et al., 1994; Aviezer et al., 1994] to elicit receptor dimerization and the subsequent tyrosine phosphorylation. The activated receptor can phosphorylate cytoplasmic proteins with SH2 (Src Homology 2) domains and a series of serine/threonine protein kinases (MAPK, MAPKK, and MAPKKK) leading to the activation of intranuclear transcription factors [Marshall, 1995].

The introduction of a hydrophilic residue in a highly hydrophobic receptor domain, as in achondroplasia, is expected to disrupt the α helical structure of the transmembrane portion and could interfere with signal transduction elicited by ligand binding. Whether such an alteration would reduce or accelerate chondrocyte differentiation and proliferation is not yet elucidated. Interestingly, FGFR-3 mutations that occurred in the extracellular receptor domain of TD I patients created new unpaired cysteine residues. We suggest that disulfide bonding between two mutant monomers could induce receptor dimerization independently of the ligand. Such an autonomous receptor activation has been observed with other tyrosine-kinase receptors including colony stimulating factor 1 (CSF-1), epidermal growth factor (EGFR) and the RET oncogene [Roussell et al., 1990; Sorokin et al., 1992; Santoro et al., 1995]. Whether stop codon mutations which are expected to give rise to an elongated receptor by 141 amino-acids also induce receptor activation is questionable. Indeed, the presence of a cysteine-rich domain in the putative additional peptide may produce constitutive receptor homodimerization, thus explaining the absence of significant radiological differences between patients carrying stop codon mutations or substitutions in the extracellular domain. Of particular interest was the recent demonstration that Fgfr-3-deficient mice generated by gene targeting have lengthening of the femora. The resulting expansion of the hypertrophic growth plate cartilage as opposed to the narrow zone of hypertrophic chondrocytes seen in cartilage of TD I patients, irrespective of the position of FGFR-3 mutations, sup-



Fig. 4. Histological section of cartilage physal growth plate from TD I case harboring a R248C mutation in the extracellular domain. The lack of chondrocyte columns is noticeable.

ports the hypothesis that FGFR-3 is a negative regulator of skeletal growth [Deng et al., 1995]. Further experiments using both transgenic animals and transfected cells are required to establish if missense mutations in FGFR-3 induce loss or gain of function of the receptor.

ACKNOWLEDGMENTS

We thank members of the French chondrodysplasia network for providing samples. This work was supported by an INSERM grant and by the European Concerted Action on Chondrodysplasias (Biomed 1, Contract BMH1-CT93-1316).

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